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(54) Map kinase kinases (MEK)

(57) A mitogen-activated protein (MAP) kinase kinase gene, tMEK2, was isolated from tomato cv. Bonny Best. By mutagenesis, a permanently-active variant, tMEK2^{MUT}, was created. Both wild type tMEK2 and mutant tMEK2^{MUT} were driven by a strong constitutive promoter, tCUPΔ, in a tomato protoplast transient expression system. Pathogenesis-related genes, PR1b1 and PR3, and a wound-inducible gene, ER5, were activated by tMEK2^{MUT} expression revealing the convergence of

the signal transduction pathways for pathogen attack and mechanical stress at the level of MAPKK. Activation of biotic and abiotic stress response genes downstream of tMEK2 occurred through divergent pathways involving at least two classes of mitogen-activated protein kinase. This study shows that tMEK2 may play an important role in the interaction of signal transduction pathways that mediate responses to both biotic (eg disease) and abiotic (wound responsiveness) stresses.

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Description

[0001] The present invention relates to a derivative of a mitogen-activated protein (MAP) kinase kinase and the use of said derivative for increasing disease resistance and enhanced stress tolerance in plants.

BACKGROUND OF THE INVENTION

[0002] Signaling mechanisms that mediate plant defense responses may be strongly conserved among plants. This is supported by the observation that several classes of R genes confer disease resistance when expressed in heterologous plant species. For instance, the tomato disease resistance gene, *Cf-9*, was shown to confer responsiveness to the fungal avirulence gene product *Avr9* in transgenic tobacco and potato (Hammond-Kosack *et al.*, 1998). Although *Cladosporium fulvum* is exclusively a fungal pathogen of tomato, a rapid hypersensitive response (HR) was induced in transgenic tobacco and potato by experimentally allowing these specific interactions to occur which then induced signaling pathways that could be common to the plants. Furthermore, the tomato disease resistance gene, *Pto*, which specifies race-specific resistance to the bacterial pathogen *Pseudomonas syringae* pv *tomato* carrying the *avrPto* gene, also increased the resistance of tomato to *Xanthomonas campestris* pv *vesicatoria* and *Cladosporium fulvum* when over expressed (Tang *et al.*, 1999). Clearly, it is the recognition of the pathogen that is unique to most plant species; whereas, the defense response is similar among them.

[0003] Considerable progress has now been made in understanding the signal transduction pathways following perception of biotic and abiotic stresses and the information is being used to develop strategies for modifying transgenic plants. Separate manipulations of the G protein pathway (Xing *et al.*, 1996, 1997) may elevate pathogen resistance or induce defense reactions in transgenic tobacco (Beffa *et al.*, 1995) and increase resistance to tobacco mosaic virus infection (Sano *et al.*, 1994). Multiple roles for MAPK (mitogen-activated protein kinase) in plant signal transduction have also been shown, including responsiveness to pathogens, wounding and other abiotic stresses, as well as plant hormones such as ABA, auxin and ethylene (Hirt, 1997; Kovtun *et al.*, 1998). MAPKK (mitogen-activated protein kinase kinase) from Arabidopsis (*AtMEK1*) and tomato (*LeMEK1*) have been shown to be induced by wounding (Morris *et al.*, 1997; Hackett *et al.*, 1998), and the maize (*ZmMEK1*) gene was induced by high salinity and cold (Hardin and Wolniak, 1998). These enzymes interact within MAP kinase pathways that are extensively used for transcytoplasmic signaling to the nucleus. In the MAPK signal transduction cascade, MAPKK (MAP kinase kinase) is activated by upstream MAP-KKK (mitogen-activated protein kinase kinase kinase) and in turn activates MAPK. The transcription of specific genes is induced by MAPK through phosphorylation and activation of transcription factors. This pathway has not yet been manipulated in plants.

SUMMARY OF THE INVENTION

[0004] The present invention relates to a derivative of a mitogen-activated protein (MAP) kinase kinase and the use of said derivative for increasing disease resistance and enhanced stress tolerance in plants.

[0005] According to the present invention it was determined that mutagenesis of a core phosphorylation site of a member of the MAPK cascade can create a permanently-active form, which stimulates both pathogen- and wound-inducible genes when introduced into plant cells.

[0006] Thus, according to the present invention there is provided a nucleic acid sequence encoding a derivative of a mitogen-activated protein kinase kinase gene from plants, wherein said derivative contains a negative charge in a core phosphorylation site of said protein kinase kinase gene.

[0007] Further according to the present invention there is provided a derivative of a mitogen-activated protein kinase kinase gene from plants, wherein said derivative contains a negative charge in a core phosphorylation site of said protein kinase kinase gene.

[0008] In a further embodiment of the present invention there is provided a cloning vector comprising a nucleic acid sequence encoding a derivative of a mitogen-activated protein kinase kinase gene from plants, wherein said derivative contains a negative charge in a core phosphorylation site of said protein kinase kinase gene.

[0009] The present invention also includes a transgenic plant comprising a nucleic acid sequence encoding a derivative of a mitogen-activated protein kinase kinase gene, wherein said derivative contains a negative charge in a core phosphorylation site of said protein kinase kinase gene.

[0010] Further, according to the present invention there is provided a method of increasing disease resistance or enhancing stress tolerance in a plant by introducing into said plant a nucleic acid sequence encoding a derivative of a mitogen-activated protein kinase kinase gene, wherein said derivative contains a negative charge in a core phosphorylation site of said protein kinase kinase gene.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

[0012] FIGURE 1 shows sequence analysis of tMEK2. FIGURE 1a shows the DNA (SEQ ID NO: 1) and deduced amino acid sequence (SEQ ID NO: 2). Roman numerals under the sequence indicate the 11 subdomains found in protein kinases. The asterisk indicates stop codon. FIGURE 1b shows the alignment of the deduced amino acid sequences from catalytic domains of MAPKK subfamily members (SEQ ID NO: 3 to 21). FIGURE 1c shows the alignment of amino acid sequences of tMEK2 with other MAPKKs between subdomain VII and VIII. Dashes represent gaps introduced for maximum matching. The amino acid residues in bold and italics between subdomain VII and VIII show putative phosphorylation sites.

[0013] FIGURE 2 shows the autophosphorylation and substrate phosphorylation activity of tMEK2. FIGURE 2a shows the autophosphorylation of tMEK2^{WT} and tMEK2^{MUT}. Recombinant GST-tMEK2^{WT} or GST-tMEK2^{MUT} proteins were incubated *in vitro* without any protein kinase substrate followed by SDS-PAGE and autoradiography. FIGURE 2b shows the phosphorylation of myelin basic protein (MBP) by tMEK2^{WT} and tMEK2^{MUT}. Recombinant GST-tMEK2^{WT} or GST-tMEK2^{MUT} proteins were incubated *in vitro* with MBP followed by SDS-PAGE and transfer to nitrocellulose. The upper panel is the autoradiography of the nitrocellulose filter. The lower panel is the immunoblot with anti-GST antibody.

[0014] FIGURE 3 shows the constructs of tMEK2^{WT} or tMEK2^{MUT} driven by the constitutive promoter tCUPΔ or control plasmid showing GUS gene driven by the constitutive promoter tCUPΔ.

[0015] FIGURE 4 shows the expression of tMEK2 in tomato leaf mesophyll protoplasts. The effect was analysed by quantitative RT-PCR following transient expression of tMEK2 in protoplasts. C1, no electroporation; C2, electroporation of control plasmid; MEK2^{WT}, electroporation of plasmid with tMEK2^{WT} driven by the tCUPΔ promoter, electroporation of plasmid with tMEK2^{MUT} driven by tCUPΔ promoter. The pathogenesis-related genes PR1b1, PR3 and Twil were tested. Tomato actin was used as an internal standard.

[0016] FIGURE 5 shows the activation of ER5 by tMEK2. FIGURE 5a shows RNA gel blot analysis of total RNA (15 μg) from leaves following wounding for the indicated time in hours, showing wound-induced activation of tMEK2 and ER5 genes. FIGURE 5b shows the activation of ER5 gene by tMEK2. The effect was analysed by quantitative RT-PCR following transient expression of tMEK2 in protoplasts. Lane settings are as described in Figure 4. Tomato actin was used as an internal standard.

[0017] FIGURE 6 shows the effect of MAPK inhibitors on tMEK2^{MUT}-induced gene activation. Kinase inhibitors at the concentration of 1 μM for staurosporine, 350 nM for SB 202190 and 1 μM for PD 98059, SB 203580 and SB 202474 were included in the protoplast incubation buffer.

[0018] FIGURE 7 shows the comparison of disease symptoms on a leaf from a wild type plant and on a leaf from tMEK2^{MUT} transformed plant.

DESCRIPTION OF PREFERRED EMBODIMENT

[0019] According to the present invention there is provided a derivative of a mitogen-activated protein kinase kinase (MAPKK). The present invention also relates to a method for increasing disease resistance and enhanced stress tolerance in plants using said derivative.

[0020] When used herein the term derivative means a modified MAPKK protein, wherein said modification includes the replacement of one or more amino acids of the wild type MAPKK with one or more other amino acids. Therefore said derivative is a non-naturally occurring variant of the wild type MAPKK.

[0021] MAPK signaling cascades are ubiquitous among eukaryotes from yeast to human (Guan, 1994) and mediate a large array of signal transduction pathways in plants (Hirt, 1997; Mizoguchi *et al.*, 1997). The cascades utilize the reversible phosphorylation of regulatory proteins to achieve rapid biochemical responses to changing external and internal stimuli. A specific MAPK is rapidly activated by pathways responding to cold, drought, mechanical stimuli and wounding (Bogre *et al.*, 1997; Jonak *et al.*, 1996; Seo *et al.*, 1995; Usami *et al.*, 1995). MAPKs are also rapidly activated by pathways responding to pathogen elicitors (Ligterink *et al.*, 1997; Suzuki and Shinshi, 1995). Other factors such as salicylic acid which is a signaling molecule in the pathogen response, may intervene in the signal cascade by transiently activating a MAPK in tobacco cells (Zhang and Klessig, 1997). MAPKK, which activates MAPK by phosphorylation in the signal cascade has been identified in Arabidopsis, tobacco, maize and tomato (Mizoguchi *et al.*, 1997; Shibata *et al.*, 1995; Hardin and Wolniak, 1998). Although phosphorylation of MAPKK by MAPKKK is the primary mechanism for initiating the signal cascade, regulation at the level of gene expression has also been implied. For instance, transcriptional activity of an Arabidopsis MAPKK, MEK1 (Morris *et al.*, 1997), and a tomato MAPKK, tMEK1 (Hackett *et al.*, 1998), was increased by wounding. Transcriptional activity of ZmMEK1, a maize MAPKK, was slightly increased in roots by high salinity and was substantially decreased by cold (Hardin and Wolniak, 1998). In this study, tomato tMEK2 mRNA accumulation was also induced by wounding of leaves but transient expression in protoplasts did not result in

the activation of the target gene ER5. This observation supported the view that biochemical activation of MAPKK by phosphorylation was the primary factor in signal transduction and that transcriptional control plays a secondary role.

[0022] Yeast and animal MAPKK are activated when serine and serine/threonine residues in the SxAxS/T motif, located upstream of the subdomain VIII are phosphorylated by MAPKKK. The putative consensus motif for characterised plant MAPKK is a S/TxXXxxS/T signature. This motif contains two additional residues when compared with the motif SxAxS/T detected in other eukaryotes. Thus, according to the present invention the use of a plant gene encoding the MAPKK is preferred to that of the yeast and animal genes, as the plant gene provides additional sites for manipulation. The plant genes also provide additional combinations of sites that can be modified according to the present invention. Thus, according to the present invention one or multiple sites of the plant gene can be modified.

[0023] According to the present invention, by creating a negative charge around a core phosphorylation site the activation by MAPKKK was not needed for MAPKK activity.

[0024] According to the present invention possible core phosphorylation sites include: serine and/or threonine sites located upstream of the subdomain VIII.

[0025] According to the present invention the creation of a negative charge around one of said core phosphorylation sites includes replacement of one or more amino acids with an amino acid selected from the group consisting of: any negatively charged amino acids. In one embodiment of the present invention said negatively charged amino acids include glutamic acid and aspartic acid.

[0026] In one embodiment of the present invention MAPKK gene, from various sources can be modified, as described above. As noted earlier MAPK signalling cascades are ubiquitous among eukaryotes from yeast to human. Suitable examples of a suitable gene that can be used according to the present invention include *Lycopersicum esculentum* cv Bonny Best, tMEK2, together with other genes available in the art, as exemplified by the following:

Arabidopsis thaliana, AtMAP2K α , (Jouannic S., Hamal A., Kreis M., Henry Y. 1996, Molecular cloning of an *Arabidopsis thaliana* MAP kinase kinase-related cDNA. Plant Physiol. 112:1397)

A. thaliana, AtMKK4, (Genbank accession number AB015315)

A. thaliana, AtMEK1, (Morris P.C., Cuerrier D., Leung L., Giraudat J. 1997, Cloning and characterisation of MEK1, an *Arabidopsis* gene encoding a homologue of MAP kinase kinase. Plant Mol. Biol. 35: 1057-1064)

L. esculentum tomato c.v. Alisa Craig, LeMEK1, (Genbank accession number AJ000728)

Zea mays, ZmMEK1, (Genbank accession number U83625)

A. thaliana, AtMAP2K β , (Genbank accession number AJ006871)

N. tabacum, NPK2, (Shibata W., Banno H., Hirano YIK., Irie K. Machida SUC., Machida Y. 1995, A tobacco protein kinase, NPK2, has a domain homologous to a domain found in activators of mitogen-activated protein kinases (MAPKKs). Mol. Gen. Genet. 246: 401-410)

A. thaliana, AtMKK3, (Genbank accession number AB015314)

D. discoideum, DdMEK1, (Nakai K., Kanehisa M. 1992, A knowledge base for predicting protein localisation sites in eukaryotic cells. Genomics 14:897-911.)

Leishmania donovani, LPK, (Li S., Wilson ME., Donelson JE. 1996, *Leishmania chagasi*: a gene encoding a protein kinase with a catalytic domain structurally related to MAP kinase kinase. Exp. Parasitol. 82: 87-96.)

Drosophila melanogaster, HEP, (Glise B., Bourbon H., Noselli S. Hemipterous encodes a novel *Drosophila* MAP kinase kinase, required for epithelial cell sheet movement. 1995, Cell 83: 451-461.)

Homo sapiens, MEK1, (Zheng C., Guan K. 1993, Cloning and characterisation of two distinct human extracellular signal-regulated kinase activator kinases MEK1 and MEK2. J. Biol. Chem. 268: 11435-11439)

R. norvegicus, MEK5, (English JM., Vanderbilt CA., Xu S., Marcus S., Cobb MH. 1995, Isolation of MEK5 and differential expression of alternatively spliced forms. J. Biol. Chem. 270: 28897-28902.)

H. sapiens, MKK3, (Derijard B., Raugeaud J., Barrett T., Wu IH., Han J., Ulevitch RJ., Davis RJ. 1995, Independent

human MAPkinase signal transduction pathways defined by MEK and MKK isoforms. Science 267:682-685.)

Saccharomyces cerevisiae, PBS2, (Boguslawski G., Polazzi JO. 1987, Complete nucleotide sequence of a gene conferring polymyxin B resistance on yeast: similarity of the predicted polypeptide to protein kinases. Proc. Natl. Acad. Sci. USA 84: 5848-5852.)

S. cerevisiae, STE7, (Teague MA., Chaleff DT., Errede B. 1986, Nucleotide sequence of the yeast regulatory gene STE7 predicts a protein homologous to protein kinases. Proc. Natl. Acad. Sci. USA 83: 7371-7375.)

Candida albicans, FIST 7, (Clark KL., Feldmann PJ. Dignard D. 1995, Constitutive activation of the *Saccharomyces cerevisiae* mating response pathway by a MAP kinase kinase from *Candida albicans*. Mol. Gen. Genet. 249: 609-621.)

S. cerevisiae, MKK1, (Irie T., Takase MKS., Lee KS., Levin DE., Araki H., Matsumoto K., Oshima Y. 1993, MKK1 and MKK2, encoding *Saccharomyces cerevisiae* MAP kinase kinase homologues function in the pathway mediated by protein kinase C. Mol. Cell. Biol. 13:3076-3083.)

[0027] In a further embodiment of the present invention putative phosphorylation activation sites are selected from the group consisting of:

Lycopersicum esculentum c.v. Bonny Best, tMEK 2: 219serine, 220threonine, 221serine and 226threonine;
Arabidopsis thaliana, AtMAP2K α : 220threonine, 226serine and 227serine;
A. thaliana, AtMKK4: 220threonine, 226serine and 227serine;
A. thaliana, AtMEK1: 219serine, 220threonine, 221serine, 222serine and 226serine;
L. esculentum, LeMEK1: 219serine, 220threonine, 221serine and 226threonine;
Zea mays, ZmMEK1: 219serine, 220serine and 226threonine;
A. thaliana, At MAP2K β : 218threonine, 220threonine and 226threonine;
N. tabacum, NPK2: 219serine, 220serine and 226threonine;
A. thaliana, AtMKK3: 220serine and 226threonine;
D. discoideum, DdMEK1, 220threonine, 222serine and 226threonine;
Leishmania donovani, LPK: 220threonine, 224serine, 225serine and 226threonine;
Drosophila melanogaster, HEP: 220serine and 226threonine;
Homo sapiens, MEK1: 220serine and 226serine;
R. norvegicus, MEK5: 220serine and 226threonine;
H. sapiens, MKK3: 220serine and 226threonine;
Saccharomyces cerevisiae, PBS2: 220serine and 226threonine;
S. cerevisiae, STE7: 220serine and 226threonine;
Candida albicans, HST 7: 220serine and 226threonine; and
S. cerevisiae, MKK1: 220serine, 225threonine and 226threonine;

wherein the amino acid numbering system is based on the tomato gene tMEK2.

[0028] In one further embodiment of the present invention, there is provided a derivative of a mitogen-activated protein kinase kinase gene from tomato cv. Bonny Best, wherein the amino acids serine221 and threonine226 have been replaced with aspartic acid.

[0029] Methods of modifying amino acid sequences are well known in the art. In general terms two primers, one for the 3' end and one for the 5' end are used to amplify the coding region. PCR-based site-directed mutagenesis was then done using the procedure as described by Higuchi (1989). Based on the sequence of the PCR product two PCR reactions are used for its mutagenesis. In PCR reaction 1, a primer containing the appropriate base substitution was used together with the 5' primer to amplify the 5' end of the coding region. In PCR reaction 2, a further primer with the appropriate base substitution was used together with the 3' primer to amplify the 3' end of the coding region. Products from both reactions were then purified and combined for 3' extension. The resulting mutant was then amplified with the original 3' and 5' primers.

[0030] The present invention also includes a suitable cloning vector containing the nucleic acid sequence encoding the derivative of the MAPK gene for transforming suitable plant recipients to increase disease resistance and enhance stress tolerance in plants. Suitable cloning vectors include any cloning vectors, Ti plasmid-derived and standard viral vectors well known in the art.

[0031] The cloning vectors can include various regulatory elements well known in the art. For example the cloning vector of the present invention can further comprise a 3' untranslated region. A 3' untranslated region refers to that

portion of a gene comprising a DNA segment that contains a polyadenylation signal and any other regulatory signals capable of effecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by effecting the addition of polyadenylic acid tracks to the 3' end of the mRNA precursor. Polyadenylation signals are commonly recognized by the presence of homology to the canonical form 5' AATAAA-3' although variations are not uncommon.

[0032] Examples of suitable 3' regions are the 3' transcribed non-translated regions containing a polyadenylation signal of *Agrobacterium* tumor inducing (Ti) plasmid genes, such as the nopaline synthase (*Nos* gene) and plant genes such as the soybean storage protein genes and the small subunit of the ribulose-1, 5-bisphosphate carboxylase (ss-RUBISCO) gene.

[0033] The cloning vector of the present invention can also include further enhancers, either translation or transcription enhancers, as may be required. These enhancer regions are well known to persons skilled in the art, and can include the ATG initiation codon and adjacent sequences. The initiation codon must be in phase with the reading frame of the coding sequence to ensure translation of the entire sequence. The translation control signals and initiation codons can be from a variety of origins, both natural and synthetic. Translational initiation regions may be provided from the source of the transcriptional initiation region, or from the structural gene. The sequence can also be derived from the promoter selected to express the gene, and can be specifically modified so as to increase translation of the mRNA.

[0034] To aid in identification of transformed plant cells, the constructs of this invention may be further manipulated to include plant selectable markers. Useful selectable markers include enzymes which provide resistance to chemicals such as an antibiotic such as gentamycin, hygromycin, kanamycin, or herbicides such as phosphorothycin, glyphosate, chlorsulfuram and the like. Similarly, enzymes providing for production of a compound identifiable by colour change such as *GUS* (β -glucuronidase), or luminescence, such as luciferase are useful.

[0035] A promoter, included in the cloning vector of the present invention, can include a constitutive promoter, which will ensure continued expression of the gene. The nucleic acid sequence encoding the derivative of the MAPK gene can also be under the control of an inducible promoter. Said inducible promoter is triggered by an induction response.

[0036] Generally speaking, an inducible promoter is a promoter that is capable of directly or indirectly activating transcription of one or more DNA sequences or genes in response to an inducer. In the absence of an inducer the DNA sequences or genes will not be transcribed. Typically the protein factor, that binds specifically to an inducible promoter to activate transcription, is present in an inactive form which is then directly or indirectly converted to the active form by the inducer. The inducer can be a chemical agent such as a protein, metabolite, growth regulator, herbicide or phenolic compound or a physiological stress imposed directly by heat, cold, salt, or toxic elements or indirectly through the action of a pathogen or disease agent such as a virus. A plant cell containing an inducible promoter may be exposed to an inducer by externally applying the inducer to the cell or plant such as by spraying, watering, heating or similar methods.

[0037] A constitutive promoter directs the expression of a gene throughout the various parts of a plant and continuously throughout plant development. Examples of known constitutive promoters include those derived from the CaMV 35S and *Agrobacterium* Ti plasmid opine synthase gene (Sanders *et al.*, 1987) or ubiquitin (Christensen *et al.*, 1992). Additionally the constitutive promoter described in WO 97/28268 published August 7, 1997.

[0038] Also considered part of this invention are transgenic plants containing the variant of the present invention. Methods of regenerating whole plants from plant cells are known in the art, and the method of obtaining transformed and regenerated plants is not critical to this invention. In general, transformed plant cells are cultured in an appropriate medium, which may contain selective agents such as antibiotics, where selectable markers are used to facilitate identification of transformed plant cells. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be used to establish repetitive generations, either from seeds or using vegetative propagation techniques.

[0039] Besides viral cloning vectors, transformation can also be accomplished by particle bombardment using the nucleic acid sequence encoding the derivative of the MAPK gene. Bombardment is a DNA delivery technique using foreign DNA particles delivered to various plant cells, tissues and species using biolistic device such as gun powder-driven biolistic device (Dupont, Wilmington, DE), gas-driven particle delivery system, microtargeting particle accelerator, an air gun apparatus (Daniell, 1997), helium blasting (Pareddy *et al.*, 1997) and instruments based on electric discharge. Transformation can also be achieved by direct uptake of *Agrobacterium* that contained foreign DNA sequence into plants via stomata in the leaves of stem or roots (Clough *et al.*, 1998).

[0040] A further aspect of the present invention is directed to the use of said nucleic acid sequence encoding the derivative of the MAPK gene to increase disease resistance or to enhance stress tolerance in plants. In this aspect of the invention the nucleic acid is introduced into the plant using any of the methods described above.

[0041] Pathogenesis-related (PR) proteins are intra- and extracellular proteins that accumulate in plant tissues or cultured cells after pathogen attack or elicitor treatment (Bowles, 1990). Using PR gene expression as a marker for the plant defence response, both PR1b1 and the chitinase gene were induced by the derivative of the MAPK gene of

the present invention.

[0042] Furthermore, according to the present invention, the transcription of the tomato ER5 gene, ZG (ABA), drought and wounding (Zegzouti *et al.*, 1997) was induced by the derivative of the MAPK gene of the present invention.

[0043] Thus, according to the present invention the derivative of the MAPK gene of the present invention can activate both pathogen- and wound-related genes.

[0044] The use of said nucleic acid sequence encoding the derivative of the MAPK gene can also be used in combination with other methods to increase disease resistance or to enhance stress tolerance in plants. These other methods could include modification of downstream components for example transcription factors and transcriptional activators. The modification of transcription factors was proven to be an effective means to improve plant stress tolerance. Overexpression of a single stress-inducible transcription factor DREB1A isolated from *Arabidopsis* improved plant drought, salt, and freezing tolerance (Masuga *et al.*, 1999). Overexpression of CBF1, an *Arabidopsis* transcriptional activator, enhanced freezing tolerance (Jaglo-Ottosen *et al.*, 1998). There is potential that modification of transcription factors or transcriptional activators downstream of MAPK in our system will enhance disease resistance and stress tolerance.

[0045] In addition there are some parallel pathways that could contribute to increased disease resistance or to enhanced stress tolerance in plants if used in combination with the modified MAPK pathway of the present invention. An example of another parallel pathway would be calcium dependent protein kinase (CDPK) (Sheen, 1996). CDPK has also been shown to act as a key mediator for cold, salt, drought, dark and ABA stresses. In addition CDPK is involved in primary defence response to pathogen attack. Overexpression of either of two different CDPKs (ATCDPK1 and ATCDPK1a) in maize protoplasts active stress signalling (Sheen, 1996). Thus the co-manipulation of the two pathways should further strengthen the defence ability of the plant.

[0046] The present invention is illustrated by the following examples, which are not to be construed as limiting.

EXAMPLES

Example 1: Isolation and Modification of tMEK2.

[0047] RNA was extracted with Extact-A-Plant™ RNA Isolation Kit (CloneTech Laboratories, Inc.) from four-week-old tomato leaves. Reverse transcription was as described in Sambrook *et al.* (1989). Cloning was carried out by PCR using Taq DNA polymerase (Life Technologies Inc.). A MAPKK gene, tMEK2, was isolated from tomato cv. Bonny Best by PCR (Figure 1a) using published MAPKK gene sequences of tomato cv. Ailsa Craig and other plant species. It shares a high level of sequence homology with MAPKKs from other species and tomato cultivars (Figure 1b) but compared with MAPKKs from mammals and yeast, tMEK2 and other plant MAPKKs have two more potential core phosphorylation sites between subdomains VII and VIII (Figure 1c).

[0048] Using PCR-assisted, site-directed mutagenesis, amino acids serine221 and threonine226 were replaced with aspartic acid (Figure 1c) creating a negative charge around the core phosphorylation site so that phosphorylation of MAPKK by upstream MAPKKK is no longer necessary for activity. Two primers (5'-end and 3'-end) that span the coding region of tomato cv Ailsa Craig LeMEK1 were used for the amplification of the MAPKK coding sequence in tomato cv Bonney Best. PCR-based site-directed mutagenesis was carried out as described before (Higuchi, 1989). Based on the sequence of the PCR product, two PCR reactions were run for its mutagenesis. In PCR reaction 1, a primer containing the base substitutions (5'GTATGTGCCGACAAA GTCATTGCCAGTCCATCTGTGCTT-GCTAGTACTGCACTCACAC3', SEQ ID NO: 22) was used together with 5'-end primer to amplify a 692 bp fragment corresponding to the 5' region of the cloned MAPKK. In PCR reaction 2, a primer containing the base substitutions (5'GTACTAGCAAGCACAGATGGACTGGCCA ATGACTTTGTGGGCACATACAACTATATGTC3', SEQ ID NO:23) was used together with 3'-end primer to amplify a 429 bp fragment corresponding to the 3' region of the cloned MAPKK. Products from PCR reaction 1 and 2 were then purified and combined for 3' extension. Mutant tMEK2 was amplified with the original 5'-end primer containing *Bam*HI and *Nco*I restriction sites, and 3'-end primer containing *Sall* and *Sma*I restriction sites. The wild type and mutagenized PCR products were purified from an agarose gel using Elu-Quik DNA Purification Kit (Schleicher & Schuell) and ligated into pre-digested pGEM-T Easy vector. The inserts were digested using *Nco*I/*Sma*I and ligated into pTZ19 tCUPA-GUS-nos3'. This derivative of tCUP promoter was created by the following modifications to the original tCUP: mutation of the sequence, 3' deletion of the sequence, nucleotide addition to the sequence, deletion of an upstream out-of-frame ATG methionine initiator codon from the sequence, deletion of the fusion protein encoded by the tobacco genomic DNA from the sequence, addition of restriction sites to the sequence. In detail, exact nucleotide changes are (numbered relative to the tCUP sequence or to the tCUPA (sequence as noted): 2084 CATATGA 2090 (NdeI recognition site beginning at 2084 underlined) in the tCUP sequence mutated to 2084 CATAGATCT 2092 (BglII recognition site beginning at 2087 underlined) in the tCUPA sequence deleting one restriction site and one upstream out-of-frame ATG methionine initiator codon while adding another restriction site and two nucleotides; 2171 AATACATGG 2179 in the tCUP sequence mutated to 2173 CCACCATGG 2181 in the tCUPA sequence

adding a Kozak consensus motif for translational initiation and an NcoI recognition site at 2176 underlined); 2181 to 2224 (relative to tCUP sequence) of tobacco genomic DNA removed from tCUPA (2183 to 2226 relative to tCUPA), deleting the 3' end of the tCUP sequence and the N-terminal fusion peptide encoded by the tobacco genomic DNA. The tCUPA-GUS-nos construct was created by fusion of the tCUPA sequence with a GUS gene and nos terminator having the sequence 2183 CTCTAGAGGAT CCCCGGGTGGTCAGTCCCTT 2213 3' (SEQ ID NO:24) to the GUS ATG at 2214 on the tCUPA sequence (see Figure 3).

Example 2: Expression and Phosphorylation Analysis of Recombinant tMEK2

[0049] For in-frame cloning with GST into the *Bam*HI/*Sal*I sites in the pGEX-4T-3 vector (Amersham Pharmacia) subcloned PCR products in pGEM-T Easy vector were digested by *Bam*HI/*Sal*I and ligated into pGEX-4T-3 cut with the same enzymes. Sequences of cloned products were confirmed by DNA sequencing. The proteins were expressed as glutathione-S-transferase fusions (GST) and purified by glutathione-agarose (Sigma) affinity chromatography essentially as described in manufacturer's protocol. Protein concentration was determined with a Bio-Rad detection system (Bio-Rad).

[0050] Autophosphorylation assay contained 1 µg of GST-tMEK2^{WT} or GST-tMEK2^{MUT} in 30 mM Hepes (pH 7.5), 5 mM of MgSO₄, 5 mM of MnSO₄, and 1 mM CaCl₂, 10 mM ATP, and 3 µCi γ-³²P-ATP (specific activity 222 TBq/mmol) in a total volume of 15 µl. The reaction mixture was incubated at 30°C for 45 min and the reaction was stopped by boiling 3 min in SDS sample buffer. As shown in Figure 2a, both wild type and mutant forms of the tMEK2 enzyme showed autophosphorylation activity.

[0051] Substrate phosphorylation assays contained 1 µg of GST-tMEK2^{WT} or GST-tMEK2^{MUT}, 2 µg of myelin basic protein (MBP, Life Technologies Inc.), 30 mM Hepes (pH 7.5), 5 mM MgSO₄ and 5 mM MnSO₄. Reactions were carried out at 30°C for 30 min. Phosphorylated products were separated by 10% SDS-PAGE, transferred to nitrocellulose and autoradiographed. Both the wild type and mutant forms of the tMEK2 enzyme phosphorylated myelin basic protein (MPB) *in vitro* (Figure 2b). Protein immunoblotting was performed as described previously (Xing *et al.*, 1996) using antiGST antibody (Amersham Pharmacia) and alkaline phosphatase-conjugated secondary antibody.

Example 3: Activation of pathogen- and wound-related genes by tMEK2

[0052] To examine the effects of tMEK2^{WT} and tMEK2^{MUT} on the activation of pathogenesis-related (PR) or other pathogen-inducible genes a tomato protoplast transient expression system was developed. Chimeric genes, tCUPA-tMEK2^{WT}-nos and tCUPA-tMEK2^{MUT}-nos, were constructed using the strong constitutive promoter, tCUPA, which was derived from the tCUP promoter as by modification of the mRNA leader sequence described above. After electroporation, transient expression of potential target genes was detected by quantitative RT-PCR. The genes analysed included PR1b1, which is activated by tomato mosaic virus (Tornero *et al.*, 1997); PR3 (chitinase), which is activated during an incompatible *C. fulvum*-tomato interaction (Danhash *et al.*, 1993); and Twi, which is a pathogen- and wound-inducible gene recently identified in tomato (O'Donnell, *et al.*, 1998).

[0053] The following procedures were used.

Protoplast isolation and transformation

[0054] Tomato (*Lycopersicon esculentum* cv Bonny Best) were grown at 80% relative humidity in peat soil in growth cabinets programmed for 16 hr days at 25°C and 8 hr nights at 22°C. Light intensity was controlled at 25 pE m⁻² S⁻¹ emitted from "cool white" fluorescent lamps (Philip Canada, Scarborough, Ontario). The youngest fully expanded leaves were surface sterilized for 5 min in 4% sodium hypochlorite and rinsed three times with sterile water. The lower epidermis was gently rubbed with Carborundum, rinsed with sterile water and leaf fragments of ca. 1 cm² were floated with exposed surface facing an enzyme solution containing 0.15% macerozyme R₁₀ (Yakult Honsha Co., Japan), 0.3% Cellulase "Onozuka" Rio (Yakult Honsha Co., Japan), 0.4 M sucrose in K3 medium (Maliga *et al.*, 1973). After overnight incubation at 30 °C, the enzyme-protoplast mixture was filtered through a 100 µm nylon sieve, centrifuged at 500 g for 5 min. and floated protoplasts were collected and washed twice with W5 medium (Maliga *et al.*, 1973). The protoplasts were kept on ice in W5 medium for 2 hr before transformation.

[0055] The protoplasts were resuspended in electroporation buffer containing 150mM MgCl₂ and 0.4 M mannitol at a density of 1x10⁶ protoplasts/ml and co-electroporated with 12-15 µg of pTZ19 carrying tMEK2 gene and pJD300 carrying luciferase gene in a total volume of 500 µl as described by Leckie (1994) with some modifications. Electroporation was performed at 200 volts and 100 µF (Gene Pulser II, Bio-Rad). Protoplasts were then allowed to recover on ice in the dark for 10 min followed by centrifugation at 500 g for 5 min. After removal of the supernatant, the protoplast pellet, with about 500 µl of buffer, was supplemented with another 500 µl protoplast incubation buffer. Protoplasts were incubated in the dark at 30°C for 24 hr.

[0056] Kinase inhibitors (CalBiochem, San Diego, CA) at the concentration of 1 μ M for staurosporine, 350 nM for SB 202190 and 1 μ M for PD 98059, SB 203580 and SB 202474, when applicable, were included in the protoplast incubation buffer. The inhibitors did not change protoplast viability (data not shown).

5 Luciferase assay

[0057] Luciferase activity in protoplasts co-electroporated with the constructs under study and luciferase DNA as an internal control were determined for evaluation of transformation efficiency. Protoplasts were lysed in 200 μ L of LUC extraction buffer (100 mM KPO_4 , 1mM EDTA, 10% glycerol, 0.5% Triton X-100 and 7 mM β -mercaptoethanol, pH 7.8).
 10 After microfuge centrifugation, the supernatant was collected and a 200 μ L aliquot of LUC assay buffer (25mM Tricine, 15 mM MgCl_2 , 5mM ATP, BSA 1mg/ml, and 5 μ L β -mercaptoethanol, pH 7.8) was added to each 20 μ L aliquot followed by 100 μ L of luciferin (0.5 mM) as substrate. The reaction was assayed in a luminometer as described (Matthews *et al.*, 1995).

15 Quantitative RT-PCR

[0058] RT-PCR was as described above. The number of PCR cycles corresponded to the high end of the range in which a linear increase in products could be detected (generally 14-16 cycles were used). Reaction products were separated on 1.0 % agarose gels. Southern blot analysis was used to estimate levels of specific amplified products.
 20 Equivalence of cDNA in different samples was verified using PCR reactions for actin. Primers were designed for PCR according to published sequences for tomato PR1b1, chitinase, Twi1, ER5 and actin (Tornerio *et al.*, 1997; Danhash *et al.*, 1993; O'Donnell *et al.*, 1998; Zegzouti *et al.* 1997; Moniz de Sa and Drouin, 1996).

[0059] Our results indicated that tomato PR1b1, chitinase and Twi1 genes were activated by tMEK2^{MUT}. This indicates that tMEK2 can mediate both pathogen and wound signals. Transient expression of the native tMEK2^{WT} gene had no effect on the expression of the three target genes (Figure 4), indicating that it is not errantly activated in the protoplast system.

Example 4: Induction of the Wound-Inducible Gene ER5

[0060] Since MAPK may be the point of convergence of the signal transduction pathways for fungal elicitors and mechanical stress (Romeis *et al.*, 1999) we also examined the induction of the wound-inducible gene, ER5 (Zegzouti *et al.*, 1997). Wounding was carried out by crushing leaves across the lamina and mid-vein using a blunt forceps. RNA was extracted after wounding for the indicated period of time. Fifteen μ g of RNA was separated per lane on a denaturing formaldehyde gel. Following transfer to nylon membranes, the blot was hybridized with radio labeled fragment of tMEK2 coding region or fragment of ER5 coding region. Autoradiography was applied to visualize the hybridization signals (Sambrook *et al.*, 1989).

[0061] Wounding of tomato leaves induced both resident tMEK2 and ER5 genes, mRNA accumulation was detectable in 30 min and lasted for at least 4 hrs (Figure 5a). Transient expression of the mutant tMEK2^{MUT} gene in tomato protoplasts also activated ER5 (Figure 5b); however, tMEK2^{WT} did not (Figure 5b), showing that elevated transcription of tMEK2 alone was not sufficient for transmitting the wound signal to ER5.

Example 5: Different MAPKs downstream of tMEK2

[0062] To study divergence of the signal pathways downstream of tMEK2 the influence of tMAPK2^{MUT} expression in tomato protoplasts was examined in the presence of a broad protein kinase inhibitor (staurosporine) and inhibitors specific to the p38 class MAPK (SB 202190 or SB 203580). Staurosporine inhibited all four genes that were previously activated by tMEK2^{MUT}; whereas, inhibitors of p38 class MAPK inhibited the PR3 and ER5 genes but not PR1b1 or Twi1. Furthermore, no effects were observed with SB202474, an inert compound acting as a negative control for MAP kinase inhibition studies, or PD 98059, an inhibitor of the MAP kinase cascade which binds to MAPKKK at a site that blocks access to activating enzymes (Alessi *et al.*, 1995). The results, shown in Figure 6, are consistent with the divergence of signal pathway downstream of tMEK2. One of these pathways could include a p38 class MAPK.

Example 6: Disease Resistance

[0063] Tomato bacterial pathogen *Pseudomonas syringae* pv *tomato* was infiltrated into tomato leaves and the effect of inoculation was recorded 7 days after inoculation. A representative comparison of disease symptoms on a leaf from a wild-type plant and on a leaf from tMEK2^{MUT} transformed plant is shown in Figure 7.

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[0109] All scientific publications and patent documents are incorporated herein by reference.

[0110] The present invention has been described with regard to preferred embodiments. However, it will be obvious to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as described in the following claims.

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Leu Val Gln His Lys Trp Thr Gly Gln Phe Phe Ala Leu Lys Val Ile
20 25 30

20

Gln Leu Asn Ile Asp Glu Ala Ile Arg Lys Ala Ile Ala Gln Glu Leu
35 40 45

25

Lys Ile Asn Gln Ser Ser Gln Cys Pro Asn Leu Val Thr Ser Tyr Gln
50 55 60

Ser Phe Tyr Asp Asn Gly Ala Ile Ser Leu Ile Leu Glu Tyr Met Asp
65 70 75 80

30

Gly Gly Ser Leu Ala Asp Phe Leu Lys Ser Val Lys Arg His Ile Ile
85 90 95

35

His Arg Asp Leu Lys Pro Ser Asn Leu Leu Ile Asn His Arg Gly Glu
100 105 110

Val Lys Ile Thr Asp Phe Gly Val Ser Thr Val Met Thr Asn Thr Ala
115 120 125

40

Gly Leu Ala Asn Thr Phe Val Gly Thr Tyr Asn Tyr Met Ser Pro Glu
130 135 140

45

Arg Ile Val Gly Asn Lys Tyr Gly Asn Lys Ser Asp Ile Trp Ser Leu
145 150 155 160

50

Gly Leu Val Val Leu Glu Cys Ala Thr Gly Lys Phe Pro Tyr Ala Pro
165 170 175

Pro Asn Gln Glu Glu Thr Trp Thr Ser Val Phe Glu Leu Met Glu Ala
180 185 190

55

Ile Val Asp Gln Pro Pro Pro Ala Leu Pro Ser Gly Asn Phe Ser Pro

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	195	200	205
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	210	215	220
10	Arg		
	225		
15	<210> 4		
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	<213> Nicotiana tabacum		
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25	Arg Ala Ile His Ile Pro Thr His Arg Ile Ile Ala Leu Lys Lys Ile		
	20 25 30		
30	Asn Ile Phe Glu Lys Glu Lys Arg Gln Gln Leu Leu Thr Glu Ile Arg		
	35 40 45		
35	Thr Leu Cys Glu Ala Pro Cys Cys Gln Gly Leu Val Glu Phe Tyr Gly		
	50 55 60		
40	Ala Phe Tyr Thr Pro Asp Ser Gly Gln Ile Ser Ile Ala Leu Glu Tyr		
	65 70 75 80		
45	Met Asp Gly Gly Ser Leu Ala Asp Ile Ile Lys Val Arg Lys Arg His		
	85 90 95		
50	Leu Val His Arg Asp Ile Lys Pro Ala Asn Leu Leu Val Asn Arg Lys		
	100 105 110		
55	Gly Glu Pro Lys Ile Thr Asp Phe Gly Ile Ser Ala Gly Leu Glu Ser		
	115 120 125		
60	Ser Ile Ala Met Cys Ala Thr Phe Val Gly Thr Val Thr Tyr Met Ser		
	130 135 140		
65	Pro Glu Arg Ile Arg Asn Glu Asn Tyr Ser Tyr Pro Ala Asp Ile Trp		
	145 150 155 160		
70	Ser Leu Gly Leu Ala Leu Phe Glu Cys Gly Thr Gly Glu Phe Pro Tyr		
	165 170 175		

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Thr Ala Asn Glu Gly Pro Val Asn Leu Met Leu Gln Ile Leu Asp Asp
180 185 190

5 Pro Ser Pro Ser Leu Ser Gly His Glu Phe Ser Pro Glu Phe Cys Ser
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10 Phe Ile Asp Ala Cys Leu Lys Lys Asn Pro Asp Asp Arg
210 215 220

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15 <211> 221
<212> PRT
<213> Arabidopsis thaliana

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1 5 10 15

25 Arg Ala Ile His Ile Pro Asn His Arg Ile Leu Ala Leu Lys Lys Ile
20 25 30

Asn Ile Phe Glu Arg Glu Lys Arg Gln Gln Leu Leu Thr Glu Ile Arg
35 40 45

30 Thr Leu Cys Glu Ala Pro Cys His Glu Gly Leu Val Asp Phe His Gly
50 55 60

35 Ala Phe Tyr Ser Pro Asp Ser Gly Gln Ile Ser Ile Ala Leu Glu Tyr
65 70 75 80

Met Asn Gly Gly Ser Leu Ala Asp Ile Leu Lys Val Thr Lys Arg His
85 90 95

40 Leu Val His Arg Asp Ile Lys Pro Ala Asn Leu Leu Ile Asn His Lys
100 105 110

45 Gly Glu Pro Lys Ile Thr Asp Phe Gly Ile Ser Ala Gly Leu Glu Asn
115 120 125

Ser Met Ala Met Cys Ala Thr Phe Val Gly Thr Val Thr Tyr Met Ser
130 135 140

50 Pro Glu Arg Ile Arg Asn Asp Ser Tyr Ser Tyr Pro Ala Asp Ile Trp
145 150 155 160

55 Ser Leu Gly Leu Ala Leu Phe Glu Cys Gly Thr Gly Glu Phe Pro Tyr
165 170 175

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5 Ile Ala Asn Glu Gly Pro Val Asn Leu Met Leu Gln Ile Leu Asp Asp
 180 195 190
 Pro Ser Pro Thr Pro Pro Lys Gln Glu Phe Ser Pro Glu Phe Cys Ser
 195 200 205
 10 Phe Ile Asp Ala Cys Leu Gln Lys Asp Pro Asp Ala Arg
 210 215 220
 15 <210> 6
 <211> 286
 <212> PRT
 <213> Dictyostelium discoideum
 20 <400> 6
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 1 5 10 15
 25 Leu Ala Tyr His Glu Thr Ser Gly Thr Tyr Ile Ala Leu Lys Val Ile
 20 25 30
 Thr Leu Asp Ile Gln Glu Asn Ile Arg Lys Gln Ile Ile Leu Glu Leu
 35 40 45
 30 Lys Thr Leu His Lys Thr Ser Tyr Pro Tyr Ile Val Ser Phe Tyr Asp
 50 55 60
 35 Ala Phe Tyr Thr Glu Gly Ser Ile Phe Ile Ala Leu Glu Phe Met Glu
 65 70 75 80
 Leu Gly Ser Leu Ser Asp Ile Met Lys Lys Thr Ser Leu His Leu Ile
 85 90 95
 40 His Arg Asp Ile Lys Pro Ser Asn Ile Leu Val Asn Asn Lys Gly Glu
 100 105 110
 45 Ala Lys Ile Ala Asp Phe Gly Val Ser Gly Gln Leu Gln His Thr Leu
 115 120 125
 Ser Lys Ala Val Thr Trp Val Gly Thr Val Thr Tyr Met Ser Pro Glu
 130 135 140
 50 Arg Ile Ser Gly Arg Ser Tyr Ser Phe Asp Ser Asp Ile Trp Ser Leu
 145 150 155 160
 55 Gly Leu Thr Ile Leu Glu Cys Ala Ile Gly Lys Phe Pro Tyr Gly Ser

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Ile Ala Asn Glu Gly Pro Val Asn Leu Met Leu Gln Ile Leu Asp Asp
180 185 190

5 Pro Ser Pro Thr Pro Pro Lys Gln Glu Phe Ser Pro Glu Phe Cys Ser
195 200 205

10 Phe Ile Asp Ala Cys Leu Gln Lys Asp Pro Asp Ala Arg
210 215 220

<210> 6
15 <211> 286
<212> PRT
<213> Dictyostelium discoideum

<400> 6
20 Leu Lys Ile Ile Arg Val Leu Gly Arg Gly Ala Gly Gly Val Val Lys
1 5 10 15

25 Leu Ala Tyr His Glu Thr Ser Gly Thr Tyr Ile Ala Leu Lys Val Ile
20 25 30

Thr Leu Asp Ile Gln Glu Asn Ile Arg Lys Gln Ile Ile Leu Glu Leu
35 40 45

30 Lys Thr Leu His Lys Thr Ser Tyr Pro Tyr Ile Val Ser Phe Tyr Asp
50 55 60

35 Ala Phe Tyr Thr Glu Gly Ser Ile Phe Ile Ala Leu Glu Phe Met Glu
65 70 75 80

Leu Gly Ser Leu Ser Asp Ile Met Lys Lys Thr Ser Leu His Leu Ile
85 90 95

40 His Arg Asp Ile Lys Pro Ser Asn Ile Leu Val Asn Asn Lys Gly Glu
100 105 110

45 Ala Lys Ile Ala Asp Phe Gly Val Ser Gly Gln Leu Gln His Thr Leu
115 120 125

Ser Lys Ala Val Thr Trp Val Gly Thr Val Thr Tyr Met Ser Pro Glu
130 135 140

50 Arg Ile Ser Gly Arg Ser Tyr Ser Phe Asp Ser Asp Ile Trp Ser Leu
145 150 155 160

55 Gly Leu Thr Ile Leu Glu Cys Ala Ile Gly Lys Phe Pro Tyr Gly Ser

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165

170

175

5

Asn Leu Pro His Gln Gln Gln Gln Pro Leu Gln Gln Gln Leu Gln Asn
180 185 190

10

Leu Asp Ile Asn Asn Ser Asn Asn Asn Ile Arg Asn Ser Asn Asn Asn
195 200 205

Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn
210 215 220

15

Asn Asn Val Leu Asp Ile Ser Asn Gly Gly Leu Val Asp Ser Gly Ser
225 230 235 240

20

Ser Val Pro Glu Gly Met Gly Phe Trp Val Leu Leu Asp Cys Ile Val
245 250 255

Lys Glu Glu Val Pro Ile Leu Pro Ser Thr Phe Ser Lys Glu Phe Arg
260 265 270

25

Ser Phe Ile Ser Glu Cys Leu Gln Lys Glu Pro Thr Glu Arg
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30

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<213> Leishmania donovani

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<400> 7
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1 5 10 15

40

Phe Ala Arg Leu Lys Asn Gly Thr Ser Ile Ala Leu Lys Arg Ile Pro
20 25 30

45

Ile Ser Ser Lys Ala His Arg Asp Glu Val Asp Arg Glu Leu Gln Val
35 40 45

Phe Met Ala Arg Ala Asp Ser Pro Tyr Val Met Asn Asn Tyr Gly Ala
50 55 60

50

Phe Trp Asp Ala Glu Asp Asp Ala Ile Val Ile Pro Met Glu Trp Met
65 70 75 80

55

Pro Tyr Thr Val Lys Asp Leu Gly Leu Phe Trp Gly Gly Lys Arg Val
85 90 95

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55

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His Arg Asp Val Lys Pro Ser Asn Ile Leu Ile Asp Glu Arg Gly Asn
 100 105 110
 5
 Ile Lys Leu Cys Asp Phe Gly Ile Ser Gly Arg Leu Val Asp Ser Lys
 115 120 125
 10
 Ala Asn Thr Arg Ala Gly Cys Ala Ala Tyr Met Ala Pro Glu Arg Ile
 130 135 140
 Asp Pro Lys Lys Pro Lys Tyr Asp Ile Arg Ala Asp Val Trp Ser Leu
 145 150 155 160
 15
 Gly Ile Thr Leu Val Glu Leu Ala Thr Ala Arg Ser Pro Tyr Glu Gly
 165 170 175
 20
 Cys Asn Thr Asp Phe Glu Val Leu Thr Lys Val Leu Asp Ser Glu Pro
 180 185 190
 Pro Cys Leu Pro Tyr Gly Glu Gly Tyr Asn Phe Ser Gln Gln Phe Arg
 195 200 205
 25
 Asp Phe Val Ile Lys Cys Leu Thr Lys Asn His Gln Asp Arg
 210 215 220
 30
 <210> 9
 <211> 234
 <212> PRT
 35
 <213> Homo sapiens
 <400> 9
 Phe Glu Lys Ile Ser Glu Leu Gly Ala Gly Asn Gly Gly Val Val Phe
 1 5 10 15
 40
 Lys Val Ser His Lys Pro Ser Gly Leu Val Met Ala Arg Lys Leu Ile
 20 25 30
 45
 His Leu Glu Ile Lys Pro Ala Ile Arg Asn Gln Ile Ile Arg Glu Leu
 35 40 45
 Gln Val Leu His Glu Cys Asn Ser Pro Tyr Ile Val Gly Phe Tyr Gly
 50 55 60
 Ala Phe Tyr Ser Asp Gly Glu Ile Ser Ile Cys Met Glu His Met Asp
 65 70 75 80
 55
 Gly Gly Ser Leu Asp Gln Val Leu Lys Lys Ala Gly His Lys Ile Met

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		85		90		95
5	His Arg Asp Val Lys Pro Ser Asn Ile Leu Val Asn Ser Arg Gly Glu	100		105		110
	Ile Lys Leu Cys Asp Phe Gly Val Ser Gly Gln Leu Ile Asp Ser Met	115		120		125
10	Ala Asn Ser Phe Val Gly Thr Arg Ser Tyr Met Ser Pro Glu Arg Leu	130		135		140
15	Gln Gly Thr His Tyr Ser Val Gln Ser Asp Ile Trp Ser Met Gly Leu	145		150		155
	Ser Leu Val Glu Met Ala Val Gly Arg Tyr Pro Ile Pro Pro Pro Asp	165		170		175
20	Ala Lys Glu Leu Glu Leu Met Phe Gly Gly Met Asp Ser Arg Pro Pro	180		185		190
25	Met Ala Ile Phe Glu Leu Leu Asp Tyr Ile Val Asn Glu Pro Pro Pro	195		200		205
	Lys Leu Pro Ser Gly Val Phe Ser Leu Glu Phe Gln Asp Phe Val Asn	210		215		220
30	Lys Cys Leu Ile Lys Asn Pro Ala Glu Arg	225		230		
35	<210> 10					
	<211> 177					
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40	<213> Rattus norvegicus					
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45	Ile Arg Tyr Arg Asp Thr Leu Gly His Gly Asn Gly Gly Thr Val Tyr	1		5		10
	Lys Ala Tyr His Val Pro Ser Gly Lys Ile Leu Ala Val Lys Val Ile	20		25		30
50	Leu Leu Asp Ile Thr Leu Glu Leu Gln Lys Gln Ile Met Ser Glu Leu	35		40		45
55	Glu Ile Leu Tyr Lys Cys Asp Ser Ser Tyr Ile Ile Gly Phe Tyr Gly	50		55		60

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Ala Phe Phe Val Glu Asn Arg Ile Ser Ile Cys Thr Glu Phe Met Asp
65 70 75 80

5 Gly Gly Ser Leu Asp Val Tyr Arg Lys Ile Leu Lys Ile Leu His Arg
85 90 95

10 Asp Val Lys Pro Ser Asn Met Leu Val Asn Thr Ser Gly Gln Val Lys
100 105 110

Leu Cys Asp Phe Gly Val Ser Thr Gln Leu Val Asn Ser Ile Ala Lys
115 120 125

15 Thr Tyr Val Gly Thr Asn Ala Tyr Met Ala Pro Glu Arg Ile Ser Gly
130 135 140

20 Glu Gln Tyr Gly Ile His Ser Asp Val Trp Ser Leu Gly Ile Ser Phe
145 150 155 160

Met Glu Leu Ala Leu Gly Arg Phe Pro Tyr Pro Gln Ile Gln Lys Asn
165 170 175

25 Gln

30 <210> 11
<211> 185
<212> PRT
<213> Homo sapiens

35 <400> 11
Leu Val Thr Ile Ser Glu Leu Gly Arg Gly Ala Tyr Gly Val Val Glu
1 5 10 15

40 Lys Val Arg His Ala Gln Ser Gly Thr Ile Met Ala Val Lys Arg Ile
20 25 30

45 Arg Ala Thr Val Asn Ser Gln Glu Gln Lys Arg Leu Leu Met Asp Leu
35 40 45

Asp Ile Asn Met Arg Thr Val Asp Cys Phe Tyr Thr Val Thr Phe Tyr
50 55 60

50 Gly Ala Leu Phe Arg Glu Gly Asp Val Trp Ile Cys Met Glu Leu Met
65 70 75 80

55 Asp Thr Ser Leu Asp Lys Phe Tyr Arg Lys Val Leu Asp Lys Asn Met
85 90 95

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5 Leu Ser Val Ile His Arg Asp Val Lys Pro Ser Asn Val Leu Ile Asn
 100 105 110
 Lys Glu Gly His Val Lys Met Cys Asp Phe Gly Ile Ser Gly Tyr Leu
 115 120 125
 10 Val Asp Ser Val Ala Lys Thr Met Asp Ala Gly Cys Lys Pro Tyr Met
 130 135 140
 Ala Pro Glu Arg Ile Asn Pro Glu Leu Asn Gln Lys Gly Tyr Asn Val
 15 145 150 155 160
 Lys Ser Asp Val Trp Ser Leu Gly Ile Thr Met Ile Glu Met Ala Ile
 165 170 175
 20 Leu Arg Phe Pro Tyr Glu Ser Trp Gly
 180 185
 25 <210> 12
 <211> 184
 <212> PRT
 <213> Saccharomyces cerevisiae
 30 <400> 12
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 1 5 10 15
 35 Lys Val Leu His Lys Pro Thr Asn Val Ile Met Ala Thr Lys Glu Val
 20 25 30
 Arg Leu Glu Leu Asp Glu Ala Lys Phe Arg Gln Ile Leu Met Glu Leu
 40 35 40 45
 Glu Val Leu His Lys Cys Asn Ser Pro Tyr Ile Val Asp Phe Tyr Gly
 50 55 60
 45 Ala Phe Phe Ile Glu Gly Ala Val Tyr Met Cys Met Glu Tyr Met Asp
 65 70 75 80
 Gly Gly Ser Leu Asp Lys Ile Tyr Asp Glu Ser Ser Glu Ile Gly His
 50 85 90 95
 Asn Ile Ile His Arg Asp Val Lys Pro Thr Asn Ile Leu Cys Ser Ala
 100 105 110
 55 Asn Gln Gly Thr Val Lys Leu Cys Asp Phe Gly Val Ser Gly Asn Leu

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	115	120	125
5	Val Ala Ser Leu Ala Lys Thr Asn Ile Gly Cys Gln Ser Tyr Met Ala		
	130	135	140
	Pro Glu Arg Ile Lys Ser Leu Asn Pro Asp Arg Ala Thr Tyr Thr Val		
10	145	150	155
	Gln Ser Asp Ile Trp Ser Leu Gly Leu Ser Ile Leu Glu Met Ala Leu		
	165	170	175
15	Gly Arg Tyr Pro Tyr Pro Pro Glu		
	180		
20	<210> 13		
	<211> 189		
	<212> PRT		
	<213> Saccharomyces cerevisiae		
25	<400> 13		
	Leu Val Gln Leu Gly Lys Ile Gly Ala Gly Asn Ser Gly Thr Val Val		
	1	5	10
30	Lys Ala Leu His Val Pro Asp Ser Lys Ile Val Ala Lys Lys Thr Ile		
	20	25	30
	Pro Val Glu Gln Asn Asn Ser Thr Ile Ile Asn Gln Leu Val Arg Glu		
35	35	40	45
	Leu Ser Ile Val Lys Asn Val Lys Pro His Glu Asn Ile Ile Thr Phe		
	50	55	60
40	Tyr Gly Ala Tyr Tyr Asn Gln His Ile Asn Asn Glu Ile Ile Ile Leu		
	65	70	75
	Met Glu Tyr Ser Asp Cys Gly Ser Leu Asp Lys Ile Leu Ser Val Tyr		
45	85	90	95
	Lys Arg Phe Val Gln Arg Gly Thr Val Tyr Lys Ile Ile His Arg Asp		
	100	105	110
50	Ile Lys Pro Ser Asn Val Leu Ile Asn Ser Lys Gly Gln Ile Lys Leu		
	115	120	125
55	Cys Asp Phe Gly Val Ser Lys Lys Leu Ile Asn Ser Ile Ala Asp Thr		
	130	135	140

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Phe Val Gly Thr Ser Thr Tyr Met Ser Pro Glu Arg Ile Gln Gly Asn
 145 150 155 160
 5
 Val Tyr Ser Ile Lys Gly Asp Val Trp Ser Leu Gly Leu Met Ile Ile
 165 170 175
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 Glu Leu Val Thr Gly Glu Phe Pro Leu Gly Gly His Asn
 180 185
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 <210> 14
 <211> 189
 <212> PRT
 <213> Candida albicans
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 <400> 14
 Leu Leu Thr Leu Lys Gln Leu Gly Ser Gly Asn Ser Gly Ser Val Ser
 1 5 10 15
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 Lys Ile Leu His Ile Pro Thr Gln Lys Thr Met Ala Lys Lys Ile Ile
 20 25 30
 30
 His Ile Asp Ser Lys Ser Val Ile Gln Thr Gln Ile Ile Arg Glu Leu
 35 40 45
 35
 Arg Ile Leu His Glu Cys His Ser Pro Tyr Ile Ile Glu Phe Tyr Gly
 50 55 60
 40
 Ala Cys Leu Asn Asn Asn Asn Thr Ile Val Ile Cys Met Glu Tyr Cys
 65 70 75 80
 Asn Cys Gly Ser Leu Asp Lys Ile Leu Pro Leu Cys Glu Asn His Lys
 85 90 95
 45
 Ile Ile His Arg Asp Ile Lys Pro Asn Asn Val Leu Met Thr His Lys
 100 105 110
 Gly Glu Phe Lys Leu Cys Asp Phe Gly Val Ser Arg Glu Leu Thr Asn
 115 120 125
 50
 Ser Leu Ala Met Ala Asp Thr Phe Val Gly Thr Ser Met Tyr Met Ser
 130 135 140
 55
 Pro Glu Arg Ile Gln Gly Leu Asp Tyr Gly Val Lys Ser Asp Val Trp
 145 150 155 160
 Ser Thr Gly Leu Met Leu Ile Glu Leu Ala Ser Gly Val Pro Val Trp
 165 170 175

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Ser Glu Asp Asp Asn Asn Asn Asp Asp Asp Glu Asp Asp
180 185

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<210> 15

<211> 187

<212> PRT

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<213> *Saccharomyces cerevisiae*

<400> 15

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Ile Glu Thr Leu Gly Ile Leu Gly Glu Gly Ala Gly Gly Ser Val Ser
1 5 10 15

Lys Cys Lys Leu Lys Asn Gly Ser Lys Ile Phe Ala Leu Lys Val Ile
20 25 30

20

Asn Thr Leu Asn Thr Asp Pro Glu Tyr Gln Lys Gln Ile Phe Arg Glu
35 40 45

25

Leu Gln Phe Asn Arg Ser Phe Gln Ser Glu Tyr Ile Val Arg Tyr Tyr
50 55 60

30

Gly Met Phe Thr Asp Asp Glu Asn Ser Ser Ile Tyr Ile Ala Met Glu
65 70 75 80

Tyr Met Gly Gly Arg Ser Leu Asp Ala Ile Tyr Lys Asn Leu Leu Glu
85 90 95

35

Arg Gly Gly Lys Lys Val Ile His Arg Asp Ile Lys Pro Gln Asn Ile
100 105 110

40

Leu Leu Asn Glu Asn Gly Gln Val Lys Leu Cys Asp Phe Gly Val Ser
115 120 125

Gly Glu Ala Val Asn Ser Leu Ala Thr Thr Phe Thr Gly Thr Ser Phe
130 135 140

45

Tyr Met Ala Pro Glu Arg Ile Gln Gly Gln Pro Tyr Ser Val Thr Ser
145 150 155 160

50

Asp Val Trp Ser Leu Gly Leu Thr Ile Leu Glu Val Ala Asn Gly Lys
165 170 175

Phe Pro Cys Ser Ser Glu Lys Met Ala Ala Asn
180 185

55

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 <211> 133
 <212> PRT
 <213> Arabidopsis thaliana

<400> 16
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Ser Ala Lys Asn Val Lys Ile Ala Asp Phe Gly Val Ser Arg Ile Leu
 20 25 30

Ala Gln Thr Met Asp Pro Cys Asn Ser Ser Val Gly Thr Ile Ala Tyr
 35 40 45

Met Ser Pro Glu Arg Ile Asn Thr Asp Leu Asn His Gly Arg Tyr Asp
 50 55 60

Gly Tyr Ala Gly Asp Val Trp Ser Leu Gly Val Ser Ile Leu Glu Phe
 65 70 75 80

Tyr Leu Gly Arg Phe Pro Phe Ala Val Ser Arg Gln Gly Asp Trp Ala
 85 90 95

Ser Leu Met Cys Ala Ile Cys Met Ser Gln Pro Pro Glu Ala Pro Ala
 100 105 110

Thr Ala Ser Gln Glu Phe Arg His Phe Val Ser Cys Cys Leu Gln Ser
 115 120 125

Asp Pro Pro Lys Arg
 130

<210> 17
 <211> 133
 <212> PRT
 <213> Arabidopsis thaliana

<400> 17
 Arg His Ile Val His Arg Asp Ile Lys Pro Ser Asn Leu Leu Ile Asn
 1 5 10 15

Ser Ala Lys Asn Val Lys Ile Ala Asp Phe Gly Val Ser Arg Ile Leu
 20 25 30

Ala Gln Thr Met Asp Pro Cys Asn Ser Ser Val Gly Thr Ile Ala Tyr
 35 40 45

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5 Met Ser Pro Glu Arg Ile Asn Thr Asp Leu Asn Gln Gly Lys Tyr Asp
50 55 60

Gly Tyr Ala Gly Asp Ile Trp Ser Leu Gly Val Ser Ile Leu Glu Phe
65 70 75 80

10 Tyr Leu Gly Arg Phe Pro Phe Pro Val Ser Arg Gln Gly Asp Trp Ala
85 90 95

15 Ser Leu Met Cys Ala Ile Cys Met Ser Gln Pro Pro Glu Ala Pro Ala
100 105 110

Thr Ala Ser Pro Glu Phe Arg His Phe Ile Ser Cys Cys Leu Gln Arg
115 120 125

20 Glu Pro Gly Lys Arg
130

25 <210> 18
<211> 133
<212> PRT
<213> Lycopersicon esculentum

30 <400> 18
Arg Arg Ile Ile His Arg Asp Leu Lys Pro Ser Asn Leu Leu Ile Asn
1 5 10 15

35 His Arg Gly Glu Val Lys Ile Thr Asp Phe Gly Val Ser Lys Ile Leu
20 25 30

40 Thr Ser Thr Ser Ser Leu Ala Asn Ser Phe Val Gly Thr Tyr Pro Tyr
35 40 45

Met Ser Pro Glu Arg Ile Ser Gly Ser Leu Tyr Ser Asn Lys Ser Asp
50 55 60

45 Ile Trp Ser Leu Gly Leu Val Leu Leu Glu Cys Ala Thr Gly Lys Phe
65 70 75 80

50 Pro Tyr Thr Pro Pro Glu His Lys Lys Gly Trp Ser Ser Val Tyr Glu
85 90 95

Leu Val Asp Ala Ile Val Glu Asn Pro Pro Pro Cys Ala Pro Ser Asn
100 105 110

55 Leu Phe Ser Pro Glu Phe Cys Ser Phe Ile Ser Gln Cys Val Gln Lys

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	115	120	125
5	Asp Pro Arg Asp Arg 130		
10	<210> 19 <211> 133 <212> PRT <213> Lycopersicon esculentum		
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20	His Arg Gly Asp Val Lys Ile Thr Asp Phe Gly Val Ser Ala Val Leu 20 25 30		
25	Ala Ser Thr Ser Gly Leu Ala Asn Thr Phe Val Gly Thr Tyr Asn Tyr 35 40 45		
30	Met Ser Pro Glu Arg Ile Ser Gly Gly Ala Tyr Asp Tyr Lys Ser Asp 50 55 60		
35	Ile Trp Ser Leu Gly Leu Val Leu Leu Glu Cys Ala Thr Gly His Phe 65 70 75 80		
40	Pro Tyr Lys Pro Pro Glu Gly Asp Glu Gly Trp Val Asn Val Tyr Glu 85 90 95		
45	Leu Met Glu Thr Ile Val Asp Gln Pro Glu Pro Cys Ala Pro Pro Asp 100 105 110		
50	Gln Phe Ser Pro Gln Phe Cys Ser Phe Ile Ser Ala Cys Val Gln Lys 115 120 125		
55	His Gln Lys Asp Arg 130		
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	<400> 20 Arg His Val Ile His Arg Asp Ile Lys Pro Ser Asn Leu Leu Val Asn 1 5 10 15		

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5 Lys Lys Gly Glu Val Lys Ile Thr Asp Phe Gly Val Ser Ala Val Leu
 20 25 30
 Ala Ser Ser Ile Gly Gln Arg Asp Thr Phe Val Gly Thr Tyr Asn Tyr
 35 40 45
 10 Met Ala Pro Glu Arg Ile Ser Gly Ser Thr Tyr Asp Tyr Lys Ser Asp
 50 55 60
 15 Ile Trp Ser Leu Gly Leu Val Ile Leu Glu Cys Ala Ile Gly Arg Phe
 65 70 75 80
 Pro Tyr Ile Pro Ser Glu Gly Glu Gly Trp Leu Ser Phe Tyr Glu Leu
 85 90 95
 20 Leu Glu Ala Ile Val Asp Gln Pro Pro Pro Ser Ala Pro Ala Asp Gln
 100 105 110
 25 Phe Ser Pro Glu Phe Cys Ser Phe Ile Ser Ser Cys Ile Gln Lys Asp
 115 120 125
 Pro Ala Gln Arg
 130
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 <210> 21
 <211> 88
 <212> PRT
 35 <213> Unknown
 <220>
 <223> Description of Unknown Organism: another MAPKK
 40 gene
 <400> 21
 45 Asp Thr Phe Thr Gly Thr Tyr Asn Tyr Met Ala Pro Glu Arg Ile Ser
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 Gly Gln Lys His Gly Tyr Met Ser Asp Ile Trp Ser Leu Gly Leu Val
 20 25 30
 50 Met Leu Glu Leu Ala Thr Gly Glu Phe Pro Tyr Pro Pro Arg Glu Ser
 35 40 45
 55 Phe Tyr Glu Leu Leu Glu Ala Val Val Asp His Pro Pro Pro Ser Ala
 50 55 60

Pro Ser Asp Gln Phe Ser Glu Glu Phe Cys Ser Phe Val Ser Ala Cys
65 70 75 80

5

Ile Gln Lys Asn Ala Ser Asp Arg
85

10

<210> 22
<211> 59
<212> DNA
<213> Artificial Sequence

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<220>
<223> Description of Artificial Sequence:primer

20

<400> 22
gtatgtgccg acaaagtcac tggccagtcac atctgtgctt gctagtactg cactcacac 59

25

<210> 23
<211> 59
<212> DNA
<213> Artificial Sequence

30

<220>
<223> Description of Artificial Sequence:primer

35

<400> 23
gtactagcaa gcacagatgg actggccaat gactttgtcg gcacatacaa ctatatgtc 59

40

<210> 24
<211> 31
<212> DNA
<213> Artificial Sequence

45

<220>
<223> Description of Artificial Sequence:nucleic acid
sequence

50

<400> 24
ctctagagga tccccgggtg gtcagtcct t 31

55

Claims

1. A nucleic acid sequence encoding a derivative of a plant mitogen-activated protein kinase kinase, wherein said

derivative contains a negative charge at a core phosphorylation site of said protein kinase kinase.

2. The nucleic acid sequence of claim 1, wherein said derivative comprises replacement of one or more amino acids with an amino acid selected from the group consisting of: aspartic acid and glutamic acid.
3. The nucleic acid sequence of claim 2, wherein said derivative comprises replacement of one or more serine or threonine amino acids with an amino acid selected from the group consisting of: aspartic acid and glutamic acid.
4. The nucleic acid sequence of claim 3, wherein said nucleic acid sequence is isolated from the group consisting of: *Arabidopsis thaliana*, *Lycopersicum esculentum*, *Zea mais*, *N tabucum*, *D discoideum* and *Leishmania donovani*.
5. The nucleic acid sequence of claim 4, wherein said one or more threonine or serine amino acids are selected from the group consisting of:

Lycopersicum esculentum c.v. Bonny Best, tMEK 2: 219serine, 220threonine, 221 serine and 226threonine;
Arabidopsis thaliana, AtMAP2K α : 220threonine, 226serine and 227serine;
A. thaliana, AtMKK4: 220threonine, 226serine and 227serine;
A. thaliana, AtMEK1: 219serine, 220threonine, 221serine, 222serine and 226serine;
L. esculentum, LeMEK1: 219serine, 220threonine, 221serine and 226threonine;
Zea mais, ZmMEK1: 219serine, 220serine and 226threonine;
A. thaliana, At MAP2K β : 218threonine, 220threonine and 226threonine;
N tabucum, NPK2: 219serine, 220serine and 226threonine;
A. thaliana, AtMKK3: 220serine and 226threonine;
D discoideum, DdMEK1, 220threonine, 222serine and 226threonine; and
Leishmania donovani, LPK: 220threonine, 224serine, 225serine and 226threonine;

wherein the amino acid numbering system is based on the tomato gene tMEK2.

6. The nucleic acid sequence of claim 5, wherein the nucleic acid is from tomato cv. Bonny Best, and wherein in the encoded derivative amino acids serine221 and threonine226 have been replaced with aspartic acid.
7. A derivative of a plant mitogen-activated protein kinase kinase, wherein said derivative contains a negative charge in a core phosphorylation site of said protein kinase kinase.
8. The derivative of claim 7, wherein said derivative comprises replacement of one or more amino acids with an amino acid selected from the group consisting of: aspartic acid and glutamic acid.
9. The derivative of claim 8, wherein said derivative comprises replacement of one or more serine or threonine amino acids with an amino acid selected from the group consisting of: aspartic acid and glutamic acid.
10. The derivative of claim 9, wherein the mitogen-activated protein kinase kinase is derived from plants selected from the group consisting of: *Arabidopsis thaliana*, *Lycopersicum esculentum*, *Zea mais*, *N tabucum*, *D discoideum* and *Leishmania donovani*.
11. The derivative of claim 10, wherein one or more of said serine or threonine amino acids are selected from the group consisting of:

Lycopersicum esculentum c.v. Bonny Best, tMEK 2: 219serine, 220threonine, 221serine and 226threonine;
Arabidopsis thaliana, AtMAP2K α : 220threonine, 226serine and 227serine;
A. thaliana, AtMKK4: 220threonine, 226serine and 227serine;
A. thaliana, AtMEK1: 219serine, 220threonine, 221serine, 222serine and 226serine;
L. esculentum, LeMEK1: 219serine, 220threonine, 221serine and 226threonine;
Zea mais, ZmMEK1: 219serine, 220serine and 226threonine;
A. thaliana, At MAP2K β : 218threonine, 220threonine and 226threonine;
N tabucum, NPK2: 219serine, 220serine and 226threonine;
A. thaliana, AtMKK3: 220serine and 226threonine;
D discoideum, DdMEK1, 220threonine, 222serine and 226threonine; and

Leishmania donovani, LPK: 220threonine, 224serine, 225serine and 226threonine;

wherein the amino acid numbering system is based on the tomato gene tMEK2.

12. The derivative of claim 11, wherein the derivative of a mitogen-activated protein kinase kinase is derived from tomato cv. Bonny Best, and wherein the amino acids serine221 and threonine226 have been replaced with aspartic acid.
13. A cloning vector comprising the nucleic acid sequence of claim 1.
14. A transgenic plant comprising the cloning vector of claim 13.
15. A transgenic plant comprising the nucleic acid sequence of claim 1.
16. A method of increasing disease resistance or enhancing stress tolerance in a plant by introducing into said plant a nucleic acid sequence encoding a derivative of a mitogen-activated protein kinase kinase, wherein said derivative contains a negative charge at a core phosphorylation site of said protein kinase kinase.
17. The method of claim 16, wherein said derivative comprises replacement of one or more amino acids with an amino acid selected from the group consisting of: aspartic acid and glutamic acid.
18. The method of claim 17, wherein said derivative comprises replacement of one or more serine or threonine amino acids with an amino acid selected from the group consisting of: aspartic acid and glutamic acid.
19. The method of claim 18, wherein said nucleic acid is isolated from the group consisting of: *Arabidopsis thaliana*, *Lycopersicum esculentum*, *Zea mais*, *N tabacum*, *D discoideum*, *Leishmania donovani*, *Drosophila melanogaste*, *Homo sapiens*, *R norvegicus*, *Saccharomyces cerevisiae* and *Candida albicans*.
20. The method of claim 19, wherein said one or more serine or threonine amino acids are selected from the group consisting of:

Lycopersicum esculentum, tMEK 2: 219serine, 220threonine, 221serine and 226threonine;
Arabidopsis thaliana, AtMAP2K α : 220threonine, 226serine and 227serine;
A. thaliana, AtMKK4: 220threonine, 226serine and 227serine;
A. thaliana, AtMEK1: 219serine, 220threonine, 221serine, 222serine and 226serine;
L. esculentum, LeMEK1: 219serine, 220threonine, 221serine and 226threonine;
Zea mais, ZmMEK1: 219serine, 220serine and 226threonine;
A. thaliana, At MAP2K β : 218threonine, 220threonine and 226threonine;
N tabacum, NPK2: 219serine, 220serine and 226threonine;
A. thaliana, AtMKK3: 220serine and 226threonine;
D discoideum, DdMEK1, 220threonine, 222serine and 226threonine;
Leishmania donovani, LPK: 220threonine, 224serine, 225serine and 226threonine;
Drosophila melanogaste, HEP: 220serine and 226threonine; human, MEK1: 220serine and 226serine;
R norvegicus, MEK5: 220serine and 226threonine;
Homo sapiens, MKK3: 220serine and 226threonine;
Saccharomyces cerevisiae, PBS2: 220serine and 226threonine;
S. cerevisiae, STE7: 220serine and 226threonine;
Candida albicans, HST 7: 220serine and 226threonine; and
S. cerevisiae, MKK1: 220serine, 225threonine and 226threonine;

wherein the amino acid numbering system is based on the tomato gene tMEK2.

21. The method of claim 20, wherein the nucleic acid is from tomato cv. Bonny Best, and wherein in the encoded derivative amino acids serine221 and threonine226 have been replaced with aspartic acid.
22. The method of claim 16, wherein said nucleic acid is introduced by a method selected from transformation and particle bombardment.

1	ATGAAGAAAGGATCTTTTGCACCTAATCTTAACTCTCTCTCTCTCTCTCTCTCTGATGAAGTT	50
1	M K K G S F A P N L K L S L P P P D E V	20
51	GETCTCTCCAAATTCCTGACTGAATCAGGAACATTTAAGGATGGAGATCTTCTGGTGAAT	120
21	A L S K F L T E S G T F K D G D L L V H	40
121	AGAGATGGAGTTGGAATTGTTTCGCAGAGTGAAGTTCCAGCTCCTTCAGTTATACAGCCA	180
41	R O G V R I V S Q S E V A A P S V I Q P	60
191	TCAGACAACCCAGTTATGCTTAGCTGATTTGAAGCAGTAAAAGTTATTGGAAAGGGAAAT	240
51	S C N Q L C L A D F E A V K V I G K G H	80
241	GGTGTATAGTCCGGCTGGTTCAGCATAAATGGACAGGGCAATTTTTCGCTCTCAAGGTT	300
91	G G I V R L V Q H K W T G Q F F A L K V	100
301	ATTTCAGATGAATATTGATGAGTCTATCCGCAACATATTGCTCAAGAACTGAGAAITTAAT	360
101	I Q H N I D E S N R K H I A Q E L R I H	120
351	CAGTCATCCCAGTGTCCATATGTTGTCATATGCTATCAGTCGTTCTTCGACAATGGTGT	420
121	Q S S Q C P Y V V I C Y Q S F F O N G A	140
421	ATATCCTTGATTTTGGAGTATATGGATGGTGGTTCCTTAGCAGATTTTTCGAAAAAGGTC	480
141	I S L I L E Y N O G G S L A D F L K K V	160
481	AAAACAATACCTGAACGATTTCTTGCTGTTATCTGCAAAACAGGTTCTCAAAGGCTTGTGG	540
161	K T I P E R F L A V I C K Q V L K G L W	180
541	TATCTTCATCATGAGAAGCATATTATTACAGGGATTTGAAACCTTCGAATTTGCTAATC	600
181	Y L H H E K N I I N R D L K P S N L L I	200
601	AATCACAGAGGTGATGTCAAATCACAGACTTTGGTGTGAGTGCAGTACTAGCAAGCACA	660
201	N H R G D V K I T D F G V S A V L A S T	220
661	TCTGGACTGGCCAATACCTTTGTGGGCACATACAATATATGTCTCCAGAGAGAATTTCA	720
221	S G L A N T F V G T Y N Y H S P E R I S	240
721	GGAGGTGCTATGATTACAAAAGCGACATTTGGAGCTTGGGTTTAGTCTTGCTCGAGTGT	780
241	G G A Y D Y K S D I W S L G L V L L E C	260
781	GCAACAGGTCATTTCCCATATAAACCCAGGGAGATGAAGGATGGGTCAATGTCTAT	840
261	A T G H F P Y K P P E G D E G W V N V Y	280
841	GAACTTATGGAAACCATAGTTGACCAACCAGAACCTTGTGCACCTCCTGACCAATTTTCT	900
281	E L H E T I V D Q P E P C A P P D Q F S	300
901	CCACAATTCTGCTCATTTCATATCTGCATGTGTCCAGAAGCACCAGAAGGACAGACTGTGG	960
301	P Q F C S F I S A C V Q K H Q K D R L S	320
961	GCAAATGATCTCATGAGTCACCCCTTTCATCACCATGTACCATGACCAGGATATCGATCTT	1020
321	A N D L H S H P F I T M Y D D Q D I D L	340
1021	GGATCTTACTTCACTTCCGAGGACCTCCATTGGCAACACTTACTGAGCTATAA	1074
341	G S Y F T S A G P P L A T L T E L	358

FIGURE 1a

[illegible]

FIGURE 1b

1. tMEK2	214	SAVLA ST SGLAN TF	227	tomato cv Bonny Best
2. AtMAP2K		SRILA Q TMDPC NS S		Arabidopsis
3. AtMKK4		SRILA Q TMDPC NS S		Arabidopsis
4. AtMEK1		SKILT ST SSLAN SF		Arabidopsis
5. LeMEK1		SAVLA ST SGLAN TF		tomato cv Ailsa Craig
6. ZmMEK1		SAVLASSIGQR DTF		maize
7. AtMAP2K		STVM TN TAGLAN TF		Arabidopsis
8. NPK2		SAGLESSIAMCA TF		tobacco
9. AtMKK3		SAGLENSMAMCA TF		Arabidopsis
10. DdMEK1		SGQLQH TL SKAV TW		<i>D. discoideum</i>
11. LPK		S-KLI Q TLAVS STY		<i>leishmania donovani</i>
12. HEP		SGRLVDSK-AN TR		<i>Drosophila</i>
13. MEK1		SGQLIDSM-AN SF		human
14. MEK5		STQLVNSI-AK TY		rat
15. MKK3		SGYLVDSV-AK TM		human
16. PBS2		SGNLVASL-AK TN		yeast
17. STE7		SKKLINSI-AD TF		yeast
18. HST7		SREL TN SLAMAD TF		<i>Candida albicans</i>
19. MKK1		SGEAVNSL-AT TF		yeast

FIGURE 1c

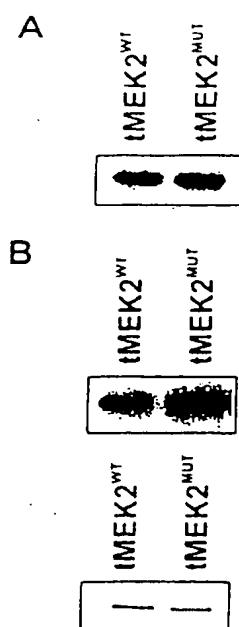


FIGURE 2

Control Construct

tMEK2^{WT} ConstructtMEK2^{MUT} Construct

FIGURE 3

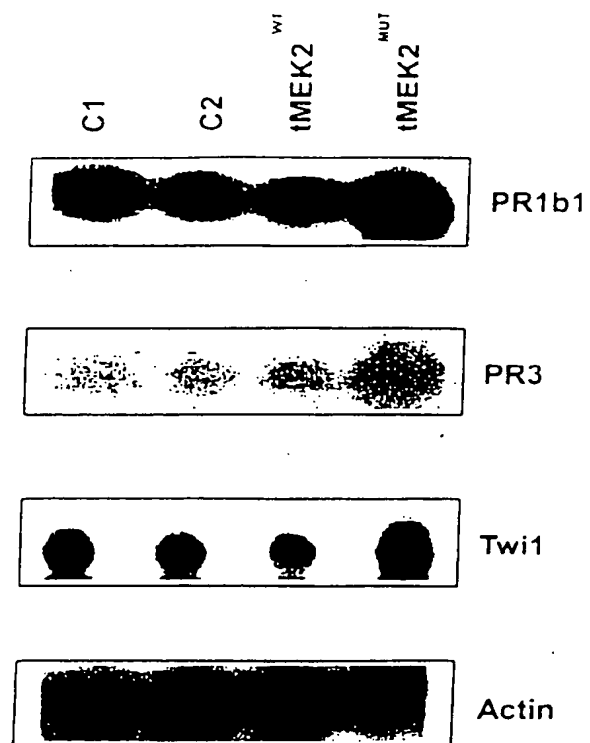


FIGURE 4

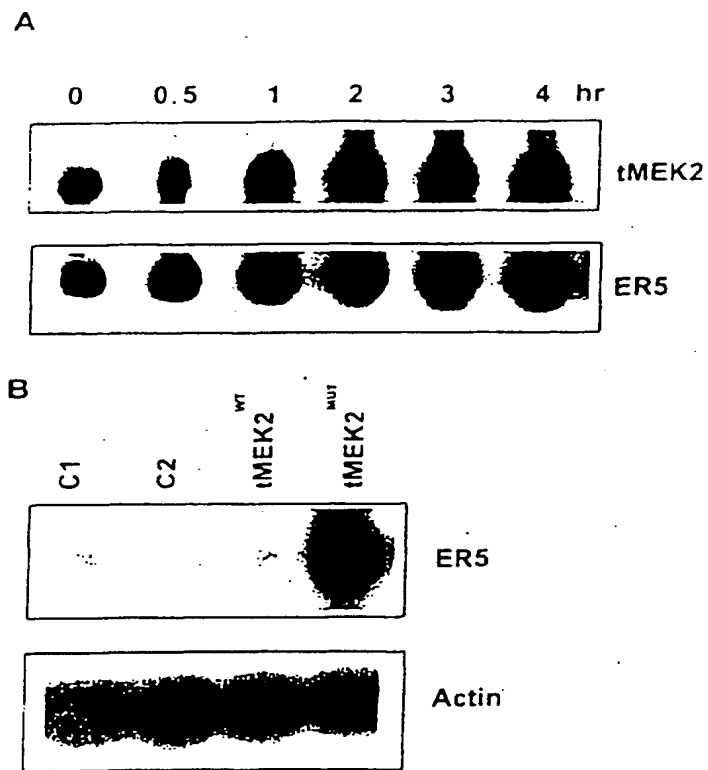


FIGURE 5

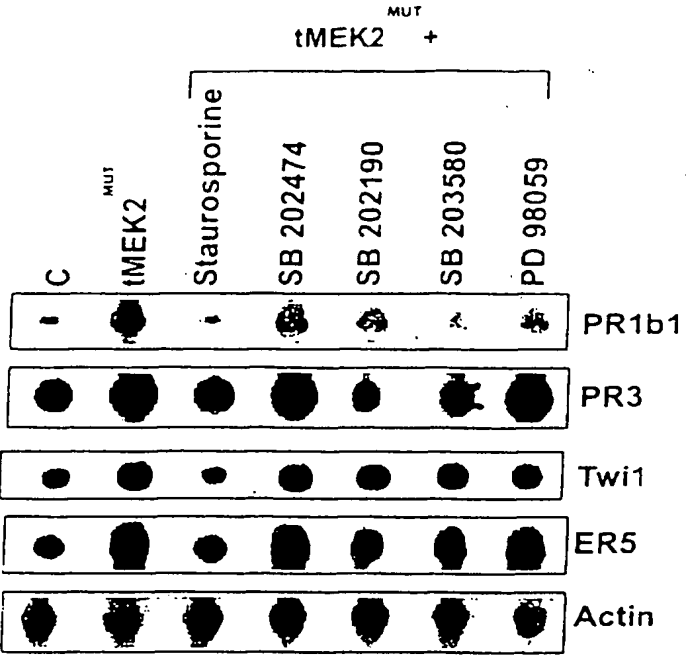


FIGURE 6



FIGURE 7

(19)



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(54) **Map kinase kinases (MEK)**

(57) A mitogen-activated protein (MAP) kinase kinase gene, tMEK2, was isolated from tomato cv. Bonny Best. By mutagenesis, a permanently-active variant, tMEK2^{MUT}, was created. Both wild type tMEK2 and mutant tMEK2^{MUT} were driven by a strong constitutive promoter, tCUPΔ, in a tomato protoplast transient expression system. Pathogenesis-related genes, PR1b1 and PR3, and a wound-inducible gene, ER5, were activated by tMEK2^{MUT} expression revealing the convergence of

the signal transduction pathways for pathogen attack and mechanical stress at the level of MAPKK. Activation of biotic and abiotic stress response genes downstream of tMEK2 occurred through divergent pathways involving at least two classes of mitogen-activated protein kinase. This study shows that tMEK2 may play an important role in the interaction of signal transduction pathways that mediate responses to both biotic (eg disease) and abiotic (wound responsiveness) stresses.

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EUROPEAN SEARCH REPORT

Application Number
EP 00 30 7362

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)		
X	MA H. ET AL.: "The Dictyostelium MAP kinase kinase DdMEK1 regulates chemotaxis and is essential for chemoattractant-mediated activation of guanylyl cyclase" THE EMBO JOURNAL, vol. 16, no. 14, 16 July 1997 (1997-07-16), pages 4317-4332, XP002156145 * page 4317, column 2, line 5 - line 14 * * page 4318, column 2, line 22-25 * * page 4318, column 2, line 47-51 * * page 4323, column 2, line 6 - page 4324, column 1, line 10; table 1 * ---	1-3,7-9, 13, 16-18,22	C12N15/11 C12N9/12 C12N15/63 C12N15/87		
D,Y	HACKETT R.M. ET AL.: "A tomato MAP kinase kinase gene (Accession No. AJ000728) differentially regulated during fruit development, leaf senescence, and wounding (PGR98-151)" PLANT PHYSIOLOGY, vol. 117, 1998, page 1526 XP000971481 * the whole document * --- -& HACKETT R.M.: "Lycopersicon esculentum mRNA for MAP kinase kinase (MEK1 gene)" EMBL DATABASE ENTRY LEAJ728 ACCESSION NO. AJ000728, 2 December 1997 (1997-12-02), XP002156146 ---	1-13	<table><tr><th>TECHNICAL FIELDS SEARCHED (Int.Cl.7)</th></tr><tr><td>C12N</td></tr></table>	TECHNICAL FIELDS SEARCHED (Int.Cl.7)	C12N
TECHNICAL FIELDS SEARCHED (Int.Cl.7)					
C12N					
D,Y					
Y	ALESSI D.R. ET AL.: "Identification of the sites in MAP kinase kinase-1 phosphorylated by p74raf-1" THE EMBO JOURNAL, vol. 13, no. 7, 1994, pages 1610-1619, XP000887212 * page 1612, column 2, line 33 - page 1613, column 1, line 15; table 1 * * page 1616, column 2, line 27 - line 34 * --- -/--	1-13			
The present search report has been drawn up for all claims					
Place of search		Date of completion of the search	Examiner		
BERLIN		7 February 2001	Schönwasser, D		
CATEGORY OF CITED DOCUMENTS					
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

EPC FORM 1503 03.82 (P04C01)



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Application Number

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CLAIMS INCURRING FEES

The present European patent application comprised at the time of filing more than ten claims.

☐ Only part of the claims have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims and for those claims for which claims fees have been paid, namely claim(s):

☐ No claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims.

LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

see sheet B

☐ All further search fees have been paid within the fixed time limit. The present European search report has been drawn up for all claims.

☐ As all searchable claims could be searched without effort justifying an additional fee, the Search Division did not invite payment of any additional fee.

☐ Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respect of which search fees have been paid, namely claims:

☒ None of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims, namely claims:

1-5,7-11,13-20,22 PARTIALLY, 6,12,21 COMPLETELY



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
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DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
A	WO 96 36642 A (DERIJARD BENOIT ; RAINGEAUD JOEL (FR); DAVIS ROGER J (US); GUPTA SH) 21 November 1996 (1996-11-21) SEQ ID NO:11	1-22	
A	NISHIHAMA R. ET AL.: "Plant homologues of components of MAPK (mitogen-activated protein kinase) signal pathways in yeast and animal cells" PLANT AND CELL PHYSIOLOGY, vol. 36, no. 5, July 1995 (1995-07), pages 749-757, XP000974668 * the whole document *	1-22	
			TECHNICAL FIELDS SEARCHED (Int.Cl.7)
<p>The present search report has been drawn up for all claims</p>			
Place of search		Date of completion of the search	Examiner
BERLIN		7 February 2001	Schönwasser, D
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

EPO FORM 1503 01.82 (PC4001)



European Patent
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LACK OF UNITY OF INVENTION
SHEET B

Application Number
EP 00 30 7362

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

1. Claims: 1-5,7-11,13-20,22 partially, 6,12,21 completely

A nucleic acid sequence encoding a derivative of a plant mitogen-activated protein kinase kinase, wherein said derivative contains a negative charge at a core phosphorylation site of said protein kinase kinase; said nucleic acid sequence, wherein one or more threonine or serine amino acids are selected from *Lycopersicon esculentum* c.v. Bonny Best, tMEK2: 219 serine, 220 threonine, 221 serine and 226 threonine; a derivative of a plant mitogen-activated protein kinase kinase, wherein said derivative contains a negative charge in a core phosphorylation site of said protein kinase kinase; said derivative, wherein one or more threonine or serine amino acids are selected from *Lycopersicon esculentum* c.v. Bonny Best, tMEK2: 219 serine, 220 threonine, 221 serine and 226 threonine; a cloning vector comprising above nucleic acid; a transgenic plant comprising said cloning vector; a transgenic plant comprising above nucleic acid; a method of increasing disease resistance or enhancing stress tolerance in a plant by introducing into said plant a nucleic acid sequence encoding a derivative of a mitogen-activated protein kinase kinase, wherein said derivative contains a negative charge at a core phosphorylation site of said protein kinase kinase and said method, wherein one or more serine or threonine amino acids are selected from *Lycopersicon esculentum*, tMEK2: 219 serine, 220 threonine, 221 serine and 226 threonine.

2. Claims: 1-5,7-11,13-22 partially

Invention no. 2 relates to subject-matter as defined above for "invention 1", with the exception, that invention no. 2 refers to AtMAP2Kalpha from *Arabidopsis thaliana* with the respective mutations at position 220 threonine, 226 serine and/or 227 serine (based on the amino acid numbering system of the tomato gene tMEK2) instead of tMEK2 from *Lycopersicon esculentum* c.v. Bonny Best.

3. Claims: 1-5,7-11,13-22 partially

Invention no. 3 relates to subject-matter as defined above for "invention 1", with the exception, that invention no. 3 refers to AtMKK4 from *Arabidopsis thaliana* with the respective mutations at position 220 threonine, 226 serine and/or 227 serine (based on the amino acid numbering system of the tomato gene tMEK2) instead of tMEK2 from *Lycopersicon esculentum* c.v. Bonny Best.



European Patent
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LACK OF UNITY OF INVENTION
SHEET B

Application Number

EP 00 30 7362

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

4. Claims: 1-5,7-11,13-22 partially

Invention no. 4 relates to subject-matter as defined above for "invention 1", with the exception, that invention no. 4 refers to AtMEK1 from *Arabidopsis thaliana* with the respective mutations at position 219 serine, 220 threonine, 221 serine, 222 serine and/or 226 serine (based on the amino acid numbering system of the tomato gene tMEK2) instead of tMEK2 from *Lycopersicon esculentum* c.v. Bonny Best.

5. Claims: 1-5,7-11,13-22 partially

Invention no. 5 relates to subject-matter as defined above for "invention 1", with the exception, that invention no. 5 refers to LeMEK1 from *Lycopersicon esculentum* with the respective mutations at position 219 serine, 220 threonine, 221 serine and/or 226 threonine (based on the amino acid numbering system of the tomato gene tMEK2) instead of tMEK2 from *Lycopersicon esculentum* c.v. Bonny Best.

6. Claims: 1-5,7-11,13-22 partially

Invention no. 6 relates to subject-matter as defined above for "invention 1", with the exception, that invention no. 6 refers to ZmMEK1 from *Zea mays* with the respective mutations at position 219 serine, 220 serine and/or 226 threonine (based on the amino acid numbering system of the tomato gene tMEK2) instead of tMEK2 from *Lycopersicon esculentum* c.v. Bonny Best.

7. Claims: 1-5,7-11,13-22 partially

Invention no. 7 relates to subject-matter as defined above for "invention 1", with the exception, that invention no. 7 refers to AtMAP2Kbeta from *Arabidopsis thaliana* with the respective mutations at position 218 serine, 220 threonine and/or 226 threonine (based on the amino acid numbering system of the tomato gene tMEK2) instead of tMEK2 from *Lycopersicon esculentum* c.v. Bonny Best.

8. Claims: 1-5,7-11,13-22 partially

Invention no. 8 relates to subject-matter as defined above for "invention 1", with the exception, that invention no. 8 refers to NPK2 from *Nicotiana tabacum* with the respective mutations at position 219 serine, 220 serine and/or 226 serine (based on the amino acid numbering system of the tomato gene tMEK2) instead of tMEK2 from *Lycopersicon*



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LACK OF UNITY OF INVENTION
SHEET B

Application Number

EP 00 30 7362

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

esculentum c.v. Bonny Best.

9. Claims: 1-5,7-11,13-22 partially

Invention no. 9 relates to subject-matter as defined above for "invention 1", with the exception, that invention no. 9 refers to AtMKK3 from Arabidopsis thaliana with the respective mutations at position 220 serine and/or 226 threonine (based on the amino acid numbering system of the tomato gene tMEK2) instead of tMEK2 from Lycopersicon esculentum c.v. Bonny Best.

10. Claims: 1-5,7-11,13-22 partially

Invention no. 10 relates to subject-matter as defined above for "invention 1", with the exception, that invention no. 10 refers to DdMEK1 from Dictyostelium discoideum with the respective mutations at position 220 threonine, 222 serine and/or 226 threonine (based on the amino acid numbering system of the tomato gene tMEK2) instead of tMEK2 from Lycopersicon esculentum c.v. Bonny Best.

11. Claims: 1-5,7-11,13-22 partially

Invention no. 11 relates to subject-matter as defined above for "invention 1", with the exception, that invention no. 11 refers to LPK from Leishmania donovani with the respective mutations at position 220 threonine, 224 serine, 225 serine and/or 226 threonine (based on the amino acid numbering system of the tomato gene tMEK2) instead of tMEK2 from Lycopersicon esculentum c.v. Bonny Best.

12. Claims: 16-20,22 partially

A method of increasing disease resistance or enhancing stress tolerance in a plant by introducing into said plant a nucleic acid encoding a derivative of a mitogen-activated protein kinase kinase, wherein said derivative contains a negative charge at a core phosphorylation site of said protein kinase kinase and said method wherein said nucleic acid is isolated from the group consisting of: Drosophila melanogaster, Homo sapiens, Rattus norvegicus, Saccharomyces cerevisiae and Candida albicans.

ANNEX TO THE EUROPEAN SEARCH REPORT
ON EUROPEAN PATENT APPLICATION NO.

EP 00 30 7362

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report.
The members are as contained in the European Patent Office EDP file on
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07-02-2001

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82